Inducible β-lactamase-mediated resistance to third-generation cephalosporins

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The emergence of multiple resistance to β-lactam antimicrobial agents is a major problem in the treatment of patients infected with Enterobacteriaceae that characteristically produce inducible β-lactamases. Inducible and 'derepressed' AmpC β-lactamases are produced by *Enterobacter* spp., *Citrobacter freundii, Serratia marcescens, Morganella morganii* and *Providencia* spp. Resistance to broad-spectrum β-lactams has emerged in 16–44% of these strains from infections treated with one of the newer cephalosporins, even in combination with other antimicrobials. Multiply resistant organisms have spread widely both locally, within hospitals, and nationally. This trend has been shown to correlate closely with the extent of usage of some third-generation cephalosporins. These resistant strains, especially *Enterobacter* spp., are more regularly isolated from seriously ill patients (especially from respiratory sources), or in intensive care units and pose one of the greatest challenges to contemporary chemotherapy of infections in hospitalized patients. Zwitterionic fourth-generation cephalosporins combine the properties of rapid bacterial outer membrane penetration with high stability to AmpC β-lactamase with good affinity for the penicillin-binding proteins to achieve in vitro activity against AmpC-producing organisms, including the majority of strains highly resistant to ceftazidime and other earlier generation cephalosporins. These features have contributed to their clinical success in the therapy of infections caused by *Enterobacter* spp. with and without resistance to third-generation compounds. Other alternative agents for chemotherapy of infections due to AmpC β-lactamase-producing strains (inducible or derepressed expression) should also be considered e.g. carbapenems, aminoglycosides and fluoroquinolones.

Key Words: AmpC, induction, derepression, cefpirome, β-lactamase

INTRODUCTION

The emergence of bacterial resistance to antimicrobial agents continues to represent an important clinical problem. In recent years, many classes of antimicrobials have become less effective as a result of evolving microbial resistance mechanisms. In some cases this has been linked to extensive use of selecting drugs [1,2]. In nosocomial infections, resistance continues to be a threat to contemporary antimicrobial chemotherapy. Current resistance problems among Gram-positive bacteria include multidrug-resistant staphylococci, glycopeptide-resistant enterococci and penicillin- and multidrug-resistant pneumococci. Resistance among Gram-negative bacteria is attributable to ceftazidime-resistant Bush Group 1 producing Enterobacteriaceae, extended-spectrum β-lactamases in *Klebsiella* spp., *Proteus mirabilis* and *Escherichia coli*, and multidrug resistance among *Pseudomonas* spp. The likelihood of encountering *Stenotrophomonas maltophilia* as a nosocomial pathogen is increasing [3]. Fluoroquinolone resistance is also present and increasing in staphylococci, enteric bacilli and *Pseudomonas* spp. [3].

Although reduced outer membrane permeability and modification of the penicillin binding proteins (PBPs) are among the most important mechanisms of bacterial resistance to β-lactam antimicrobial agents, β-lactamase production accounts for a major source of resistance [4]. Virtually all bacteria produce chromosomally-mediated β-lactamases and plasmid-mediated β-lactamases are

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widespread in Gram-negative bacteria. More recently, the plasmid-mediated, extended-spectrum β-lactamases have emerged as clinically important resistance determinants in the Enterobacteriaceae [4–6]. Metallo β-lactamases confer resistance to carbapenems and, although still uncommon at present, may pose a threat in the future [4].

The introduction of third-generation cephalosporins improved the effectiveness of therapy for the vast majority of infections caused by Gram-negative bacteria; however, the use of these highly β-lactamase stable compounds has led to the emergence of resistant species [2]. Bacteria that possess chromosomally-mediated Bush Group 1 β-lactamase have been implicated in the development of resistance and multiply-resistant, stably derepressed mutants have emerged during therapy [7,8].

Beta-lactamases are present in virtually all Gram-negative bacilli. However, in some bacterial strains, such as *E. coli* and *Klebsiella* spp., the β-lactamase is produced at a low level and cannot be induced to greater production by the presence of β-lactams. In other species, β-lactamase production occurs at low levels, but is inducible when exposed to certain β-lactams, commonly resulting in resistance to these agents. These inducible β-lactamases are frequently found in *Enterobacter* spp., *Citrobacter freundii, Providencia* spp., *Morganella* spp. and *Serratia* spp. (Table 1) [7,8]. These organisms also routinely undergo spontaneous mutation to become constitutive β-lactamase producers. This, in turn, confers resistance to most β-lactams, including third-generation cephalosporins. However, β-lactam antimicrobial agents differ, not only in their sensitivity to these enzymes, but also in their ability to induce synthesis of the enzyme and selection of resistant, derepressed mutants [4,9].

Beta-lactamases are an enormously varied class of enzymes, classified until recently both on the basis of their substrate hydrolytic spectrum and whether encoded by plasmid- or chromosomally-located genes [10–12]. However, such phenotypic classification schemes were found to be compromized in satisfactorily recognizing the point mutations which could dramatically alter substrate specificity and inhibitor susceptibilities. Therefore, β-lactamases are increasingly classified at a molecular level on the basis of amino acid sequence [13], as originally proposed by Ambler [14]. Four classes are recognized under this scheme: classes A, C and D are serine active site enzymes, whereas class B metallo-enzymes require zinc for activity. Expression of β-lactamase can be constitutive or inducible. Constitutive and non-induced enzyme levels are normally quite low; however, induction can lead to several hundred-fold increases in activity. Mutations in genetic control mechanisms can also result in derepression of the enzyme, whereby β-lactamase production is maintained at a very high level.

The genetics of induction are discussed below; however, it is important at this stage to define the terms 'induction' and 'derepression'. 'Induction' is defined as the synthesis of enzyme-protein in direct response to induction by the substrate (inducer), a phenotypic, temporary response to an environmental change. 'Derepression', in contrast, is a constitutive, permanent feature of the mutant (stable genetic change) whereby large amounts of enzyme-protein are produced consistently.

Ambler Class A β-lactamases, including the common plasmid-mediated TEM enzymes, are produced constitutively by *K. pneumoniae, Bacteroides fragilis* and inducibly by *K. oxytoca* and *Staphylococcus aureus.* Class B metallo-enzymes are relatively uncommon and mainly produced by *S. maltophilia, Bacillus cereus* and some strains of *Bacteroides* spp. However, the Class B enzymes have the important ability to rapidly hydrolyze those drugs generally stable to the other enzyme classes, such as the carbapenems and the cephamycins.

AmpC β-lactamases are produced by many bacterial species. Production of inducible AmpC β-lactamases is limited to a group of organisms including *Enterobacter* spp., *C. freundii, S. marcescens, M. morganii, Providencia* spp. and *P. aeruginosa.* These bacterial species are regularly isolated from hospitalized patients, including the seriously ill, and pose one of the greatest challenges to contemporary nosocomial or hospitalized patient infection chemotherapy [2,4,7,8,15].

Table 1 Enterobacteriaceae species often possessing inducible Bush Group 1 β-lactamases and associated with strains having resistance to so-called 'third-generation' cephalosporins

Genus	Species
Enterobacter	*aerogenes, cloacae*
Citrobacter	*freundii*
Serratia	*marcescens*
Morganella	*morganii*
Providencia	*rettgeri, stuartii*

Enterobacter spp. are increasing in clinical practice [3,16,17]. In a survey conducted in the USA in 1994, of > 8,500 organisms isolated from patients residing in 43 medical centers, *Enterobacter* spp. were responsible for 6.3% of all infections [3]. *Enterobacter* was found to be the fourth most prevalent genus in respiratory tract infections, accounting for 9.2% of infections (with *S. marcescens* accounting for a further 4.6% of infections). Of the 3,224 organisms isolated from urinary tract infections, 4.7% were *Enterobacter* spp. and 1.8% were *C. freundii*. *Enterobacter* was also a significant pathogen in skin and soft tissue infections, accounting for 6.8% of the total number of isolates (with *S. marcescens* responsible for another 2.4%). In blood stream infections, *Enterobacter* spp. accounted for 3.9% of the total. Similar data were obtained in the 1995/96 SCOPE study, where *Enterobacter* spp. and *S. marcescens* accounted for 5% and 2%, respectively, of nosocomial blood stream infections [16].

Enterobacter spp. was also found to be a significant pathogen isolated in Intensive Care Units (ICUs). In the National Nosocomial Infections Surveillance system (NNIS) of ICU infections conducted in 1990, *Enterobacter* spp. was among the top five pathogens [17]. In this study, the incidence per site of infection was: respiratory tract (5.3%), surgical wound (10.3%) and urinary tract (6.1%). These findings were confirmed by the results of a European study, where *Enterobacter* spp. accounted for 8% of pathogens isolated from infections in medical ICUs, surgical ICUs and hematology/oncology units [18].

GENETICS OF INDUCIBLE AmpC EXPRESSION IN ENTEROBACTERIACEAE

Translation of the *ampC* gene is regulated by the *ampR* gene product, AmpR [19]. AmpR is a bifunctional protein, being a transcriptional activator in the presence of some β-lactams and a repressor in their absence. Deletion mutations of *ampR* generate a non-inducible phenotype, with AmpC being expressed at a level two- to three-fold higher than the normal, uninduced basal level [20,21].

At least two other genes, *ampD* and *ampG*, are involved in AmpC induction. A third gene, *ampE*, was initially thought to be involved in β-lactamase expression, but recent work has shown that it is not required [22]. *AmpD* and *ampG* are present in all Enterobacteriaceae tested to date, even those lacking an inducible AmpC β-lactamase, suggesting other primary functions for AmpD and AmpG [23]. AmpD is, in fact, a cytosolic *N*-acetyl muramyl-L-alanine amidase which participates in the intracellular recycling of peptidoglycan fragments [23,24]. DNA protection studies have failed to show binding to the regulatory region upstream from *ampC*; hence, it is unlikely that AmpD directly influences the expression of *ampC*. Null mutations in *ampD* cause derepression, while other mutations generate a hyper-inducible phenotype, whereby lower levels of inducer are required to promote *ampC* expression.

AmpG is believed to be a permease for a large muropeptide which might be a hypothetical activating ligand for β-lactamase induction [25]. In the absence of this protein no induction occurs, nor does constitutive activation of *ampC* take place in *ampG*, *ampD* double mutants [26,27].

Several models have been proposed to show the interaction of the various genes and gene products involved in AmpC induction. New insights into the relationship between β-lactamase induction and peptidoglycan recycling have given rise to an alternative view of the Bennett and Chopra model [28]. This suggests that AmpR controls β-lactamase production by sensing the cytoplasmic level of muropeptides, which is influenced by the activities of AmpD and AmpG in peptidoglycan recycling and indicative of the presence or absence of β-lactam antimicrobials (Figure 1) [25,29,30]. Peptidoglycan recycling has a signalling role in β-lactamase induction and derepression and is part of a communication link between the dynamic state of the cell wall, essential for growth and cell division, and the transcription mechanism of *ampC*.

RESISTANCE AMONG ENTERIC BACTERIAL SPECIES

The clinical and epidemiological importance of inducible β-lactamases and their stably derepressed mutants in Gram-negative bacteria has increased dramatically since the introduction of the third-generation cephalosporins [31]. These stably derepressed mutants were present in significant numbers among clinical isolates even before the clinical introduction of the third-generation cephalosporins. Occurrence rates of more than 10% for high β-lactamase-producing strains (derepressed AmpC) among Enterobacteriaceae were not uncommon between 1976 and 1981, although the incidence of such strains varied according to site of infection, geographical location and selective pressures [32]. In 1982, before the introduction of third-generation cephalosporins, *E. cloacae, C. freundii* and *S. marcescens* isolated from medical centers in the USA were all relatively susceptible to cefotaxime, with MIC_{90} values ≤ 5 mg/L [32]. In contrast, data reported from Europe and the Far East showed that strains of *C. freundii* and *E. cloacae* were more resistant, with

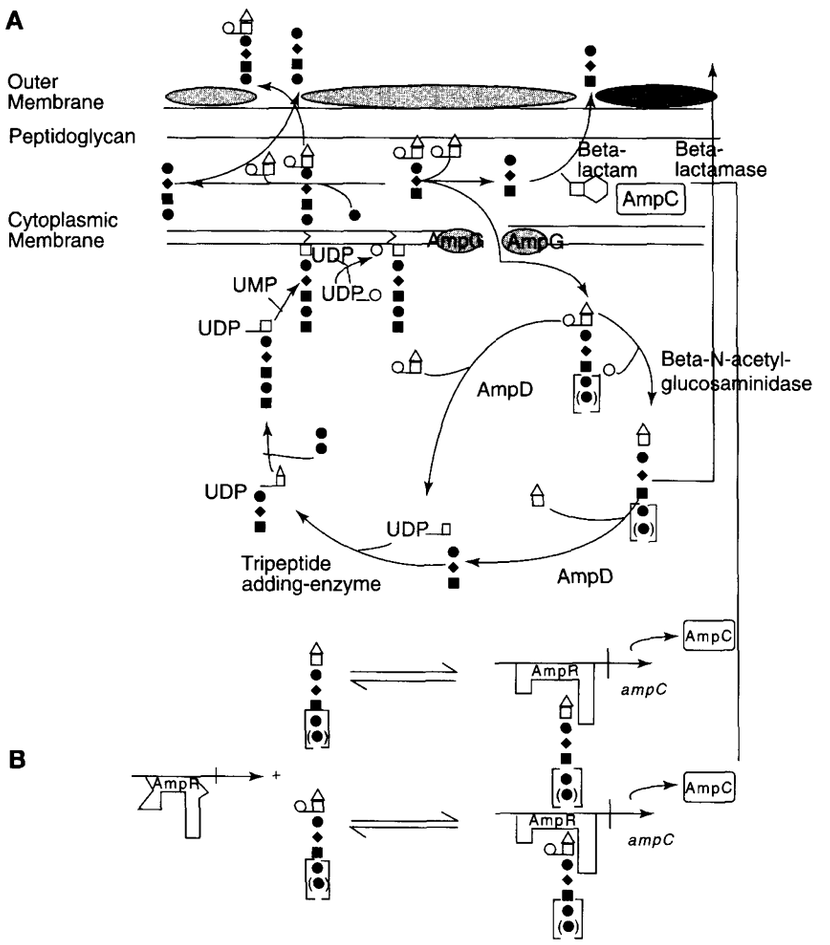

Figure 1 Hypothetical model for control of the expression of inducible *ampC* genes in Gram-negative bacteria [24–26,29,30]. The proposed interconnected pathway for recycling muropeptides and for their involvement in β–lactamase induction is illustrated. ○ GlcNac; ⌂ anhMurNac; □ MurNac; ● Ala; ◆ Glun; ■ DAP.

(A) The recycling pathway. As shown, murein is degraded by known enzymes in the periplasm to muramyl peptides. The muropeptide GlcNac-anhMurNac-tripeptide, tetrapeptide and pentapeptide are transported into the cytoplasm through AmpG. Disaccharides are cleaved by β-*N*-acetyl-glucosaminidase into monosaccharides. The muropeptides are then degraded into GlcNac-anhMurNac and free tripeptide, tetrapeptide or pentapeptide by AmpD. Free tripeptide can then be added directly to UDP-*N*-acetylmuramic acid by an as yet unidentified enzyme, thereby reintroducing it into the biosynthetic pathway for murein synthesis.

Derepression can occur because of mutations in the *ampD* or *ampR* gene. The *ampR* mutations change the AmpR protein into an activator. The *ampD* mutations alter the AmpD protein to an inactive enzyme, which results in an accumulation of the muramyl peptides in the cytoplasm.

(B) Muropeptides as inducers of β-lactamase. Intracellular accumulation of GlcNac-anhMurNac-tripeptide as a result of the presence of the β-lactam antibiotics or of anhMurNac-tripeptide as the result of inactivation of *ampD* triggers production of *C. freundii* AmpC β-lactamase. The muropeptides presumably bind to the transcriptional regulator AmpR and convert it into an activator for *ampC* expression. (*C. freundii ampR* and *ampC* are expressed from a plasmid.)

MIC_{90} values three- and 30-fold higher, respectively, clearly attributable to derepressed AmpC production (Table 2). Further reports [33–35] have also indicated that up to 40% of isolates (1987–91) had stably derepressed β-lactamases.

Over the following decade, with increased use of broad-spectrum β-lactams, resistance levels rose markedly throughout the world in general such as *Enterobacter* and in *C. freundii*, although there continued to be regional and national differences. International variations in resistance to third-generation cephalosporins have been documented in a review of surveys between selected hospitals in five nations (USA, France, Germany, Italy and Japan) [36]. Cefotaxime, used as an index third-generation cephalosporin, had relatively high susceptibilities in Germany, where 80% and 100% of *E. cloacae* and *S. marcescens* were inhibited by ≤ 8mg/L. In contrast, high levels of resistance were observed in Japan and Italy, where only 57.7% of *E. cloacae* (Japan) and 63.3% of *S. marcescens* (Italy) were susceptible.

High levels of resistance to the third-generation cephalosporins have also been reported from studies in the USA. In one report in 1993, using reference NCCLS tests and breakpoint criteria, only 66–82% of some Enterobacteriaceae remained susceptible to cefotaxime [37]. In another US survey involving >30,000 enteric bacilli isolated during 1994, 18% of *S. marcescens*, 23% of *C. freundii* and 34% of *E. cloacae* were resistant to cefotaxime. These resistance levels have been confirmed by more recent surveillance data [36–38]. Data from 1994–1995, using two standardized methods, indicated that 21–40% of *E. cloacae* isolated from blood, lower respiratory tract, urinary tract and skin and soft tissue infections were resistant to ceftazidime [3,38] (Table 3). In 1995, in a five-hospital study (>1,000 strains/site), 20–30% of strains (depending on the species tested) were resistant to the third-generation cephalosporins [39].

Resistance levels have also increased in other areas of the world, although the incidence varies according to geographic location, the testing method and interpretation criteria used. In a Belgian study conducted in 1993, the susceptibility of 8,625 ICU and hematology patient isolates was examined. Of these, 30% of *E. cloacae* and 41% of *C. freundii* strains were found to be resistant

Table 2 Activity of cefotaxime against Bush Group 1 (Ambler class A) β-lactamase-producing Enterobacteriaceae isolated prior to its widespread clinical use[a]

Organism	Collection source (no. tested)	MIC (mg/L)[b]		No. of refs. cited
		MIC_{50}	MIC_{90}	
Citrobacter freundii	USA (48)	0.11	5.0	4
	World (88)	0.35	18.2	
Enterobacter aerogenes	USA (152)	0.12	1.0	8
	World (42)	0.20	6.3	
Enterobacter cloacae	USA (153)	0.12	1.3	10
	World (245)	6.2	37.0	
Serratia marcescens	USA (597)	0.47	5.2	25
	World (449)	0.82	5.2	

[a]Modified from report of 15,672 enteric bacilli by Jones and Thornsberry [32].
[b]The MIC_{50} and MIC_{90} are the lowest concentration inhibiting growth of 50% and 90% of tested strains, respectively.

Table 3 Rates of resistance to third-generation cephalosporins (ceftazidime) among *E. cloacae* isolates reported in the USA in 1994–1995 [3,38][a]

	Monitored centers (No.)	% susceptible by infection site[b,c]			
		Blood	LRTI	UTI	SSTI
Jones et al. (1995)	43	60	66	74	71
Baron and Jones (1995)	236	75	75	ND	79[c]

[a]Data derived from NCCLS standardized test (disk diffusion and broth microdilution).
[b]Blood = bacteremias; LRTI = lower respiratory tract infections; UTI = urinary tract infections; SSTI = skin and soft tissue infections; ND = Not determined.
[c]Isolates from intra-abdominal and gynaecology wound infections exhibited 74–76% susceptibility to ceftazidime.

to third-generation cephalosporins [18], data very similar to those reported in North America.

The increase in resistance amongst Enterobacteriaceae has been correlated with an increase in the use of broad-spectrum antimicrobial agents. For example, resistance amongst *E. cloacae* to ceftazidime has been shown to be directly related to the use of ceftazidime (Figure 2) [2]. As the use of ceftazidime increased steadily, the susceptibility to ceftazidime declined ($p < 0.02$). To examine temporal trends in ceftazidime resistance, susceptibility data reported to the NNIS survey (CDC) during 1987–1991 were analyzed among nosocomial *Enterobacter* spp., *K. pneumoniae* and *P. aeruginosa*. Progressive increases in resistance were observed for *Enterobacter* spp. and *K. pneumoniae* over time, with the percentage of resistant strains of *Enterobacter* spp. increasing significantly during 1989–1991 [35]. The increase in ceftazidime resistance in *K. pneumoniae* was related to plasmid-mediated extended spectrum β-lactamases [4–6,12,13].

Resistance to third-generation agents caused by derepressed species appears to be greatest amongst the most seriously ill patients, such as those in the ICU setting [40]. Furthermore, *E. cloacae* consistently has the highest rates of resistance (ceftazidime) in general practice (GP) patients, hospitalized patients and those within the ICU (Figure 3; personal communication from the Paul Ehrlich Society, B. Wiedermann).

Resistance development may be particularly devastating in patients with serious infections, e.g. neutropenic and immunocompromized patients, especially if prior antimicrobial therapy has been given. Numerous cases of breakthrough bacteremia with multiply-resistant *Enterobacter* spp. in febrile neutropenic cancer patients and other patients receiving broad-spectrum cephalosporins have been reported [34]. The results of studies that have assessed the rates of resistance emerging among Enterobacteriaceae during or shortly after therapy with a number of cephalosporins are listed in Table 4 [8]. Resistance emerged in 16–44% of treated

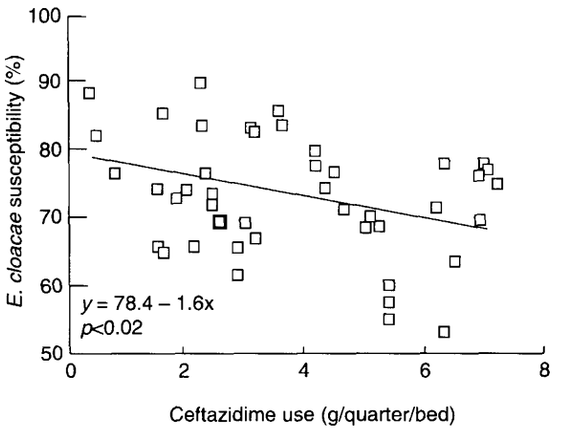

Figure 2 Relationship between ceftazidime use and susceptibility of *Enterobacter cloacae* to ceftazidime [2]. With permission of Diagn Microbiol Infect Dis.

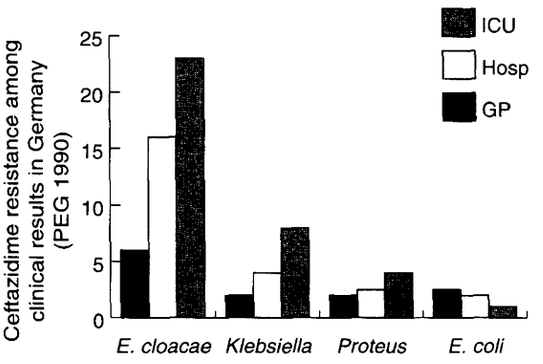

Figure 3 Ceftazidime resistance among clinical isolates in Germany (PEG 1990).

Table 4 Rates of emergence of resistance in patients infected with Enterobacteriaceae organisms possessing inducible β-lactamases and treated with newer cephalosporins. Adapted from Sanders et al. with permission [8].

Drug	Organism[a]	Total no. of patients	No.(%) of patients with emerging resistance	Frequency of clinical failure or relapse[b]
Ceftriaxone	Several	29	8 (28)	6 (21/75)
Moxalactam	*Serratia marcescens*	10	3 (30)	1 (10/33)
Moxalactam	Several	10	4 (40)	1 (10/25)
Several	*Enterobacter* species	9	4 (44)	–
Several	Several	44[c]	7 (16)[c]	3 (7/43)[c]

[a]Data summarized for enteric bacilli from four earlier publications (102 patients, not all of whom received a cephalosporin).
[b]Results are expressed as the number of patients with therapy failure or relapse (percentage of total number of patients/percentage of those with emerging resistance). Minus signs indicate that no data were provided.
[c]Includes *P. aeruginosa* (24 of 49 strains in 44 patients). Only one of the resistant enteric bacilli cases received an extended spectrum β-lactam.

patients (highest among *Enterobacter* spp.), with a mean rate of 25%. The rates were generally consistent among the various drugs examined. A more comprehensive review by Fish et al. documented a lower rate of emerging resistance (7.7–10.1%) for *Citrobacter* spp. and *Enterobacter* spp. [41]. Among patients in whom the emergence of resistance was detected, failure/relapse rates ranged from 25% to 75%, but emerging resistance did not predict clinical failure. The greatest risk of resistance and frequency of pathogen occurrence appear to occur with isolates of *E. cloacae* and *E. aerogenes*, especially those cultured from respiratory tract sites (Tables 1 and 4). High morbidity and mortality cases were also associated with bone and joint infections and in patients with neutropenia and cystic fibrosis [9]. In one investigation, 15 of 16 isolates of *Enterobacter* spp. from neutropenic patients were resistant to extended-spectrum cephalosporins. In contrast, only 12 of 35 isolates from non-neutropenic patients were resistant ($p < 0.05$) [34]. The neutropenic patients had received more β-lactam therapy than the non-neutropenic patients. The authors concluded that prior β-lactam exposure may predispose neutropenic patients to develop resistant *Enterobacter* bacteremia. Other studies have described patients where cephalosporin-resistant Gram-negative bacteria have emerged during treatment, resulting in life-threatening secondary infections [8,31,33]. A total of 18 patients who were infected initially with susceptible organisms exhibited emergence of resistant strains during administration of ceftriaxone, cefotaxime or ceftazidime, some despite combination therapy with aminoglycosides [33]. Resistant strains of *E. cloacae*, *S. marcescens*, *K. oxytoca*, *P. aeruginosa* and *C. freundii* emerged, probably by the selection of stably derepressed mutants, after 9 days of treatment. Thus, the selection of resistant bacteria may have serious clinical consequences in patients with risk factors, such as impaired host-defence mechanisms, as the selection of resistance is associated with a significant rate of therapy failure and relapse.

Risk of AmpC induction

The extent of AmpC induction is dependent upon both the β-lactam-inducing agent and the inducer concentration [9,42–45]. At sub-MIC concentrations, cefoxitin, long regarded as a potent inducing agent, has been shown to induce AmpC by 100- to 600-fold in strains of *E. cloacae*, *C. freundii*, *P. stuartii*, *S. marcescens*, *M. morganii* and *P. aeruginosa* [44]. However, the carbapenems, imipenem and meropenem, may prove to be at least as potent as cefoxitin as inducing agents for AmpC in *C. freundii* [9].

A consensus of published reports ranks the AmpC inducing potential for β-lactam classes [42–45]. On this basis, carbapenems and cephamycins are the most potent inducing agents (Table 5), followed by penicillins and the older cephalosporins. The fourth-generation cephalosporins, cefpirome and cefepime, have a lower risk of inducing AmpC than the β-lactamase inhibitor, clavulanic acid. Induction itself, however, does not imply a clinical risk, since the greatest inducers produce increased amounts of enzyme without a significant effect on the initial MIC (i.e. rapid bactericidal action becomes manifest before induction of the enzyme has been efficiently produced).

Risk of AmpC selection

Some β-lactam antimicrobials are more likely than others to select mutant subpopulations of resistant organisms and their widespread use in the hospital environment has resulted in the emergence of clinically important endemic bacterial resistances [46]. These selection potential differences in individual inducible strains that cause infection (susceptible by reference test) remains unclear.

The frequency of stably derepressed AmpC mutants in a bacterial population can be as high as 10^{-5} [4]. Such mutants have serious clinical implications and are isolated in approximately 20% of infections involving AmpC-producing strains during selective therapy with broad-spectrum β-lactams [4]. Factors favoring the

Table 5 Induction potential at concentrations below MIC (consensus from the reported literature [42–45])

Induction Potential	Rank
Highest	carbapenems and cephamycins
	aminopenicillins
	carboxy-penicillins
	ureidopenicillins
	older cephalosporins (1st, 2nd and 3rd)
	clavulanic acid
	newer cephalosporins (4th)
	sulphones
Lowest	monobactams

occurrence and selection of such mutants include high bacterial inoculum at the infection site, bacterial species and strain involved.

In an in vitro investigation of resistance development to third- and fourth-generation cephalosporins in 10 strains of *E. cloacae*, full resistance to ceftriaxone and ceftazidime occurred in at least half of the strains within 1–3 days of passage (Figure 4) [46]. This resistance development was associated with greatly enhanced AmpC production, but had only a modest effect upon outer-membrane protein profile as a resistance mechanism. In contrast, at least five passages were required before the majority of strains acquired resistance to fourth-generation cephalosporins. Resistance to the fourth-generation cephalosporins was associated with changes in the outer membrane proteins, but involved little alteration of AmpC expression. The latter results suggest that at least two genetic mutations, altered permeability and high Km, may be necessary to achieve resistance to newer zwitterionic cephalosporins.

The dramatic impact of inducible AmpC β-lactamase-producing strains upon β-lactam susceptibility and clinical outcome makes it essential that clinical microbiology laboratories can identify such strains reliably. The primary difficulties caused by Gram-negative pathogens with inducible β-lactamases stem from their apparent susceptibility, when tested against third-generation cephalosporins, in routine in vitro tests. However, accurate bacterial identification should be sufficient to raise the possibility of selecting derepressed AmpC mutants. Identification of the 'at risk' species is well within the specifications of most commonly used commercial kits (Vitek, MicroScan, Sensident, Micronaut, API, etc.). Information provided by computerized 'Expert Systems' for the interpretation of antimicrobial susceptibility testing, frequently coupled with the above cited commercial diagnostic systems, may also be useful. As confirmation, standardized susceptibility tests can accurately determine β-lactam susceptibility for the selected derepressed mutants without the need for elaborate or time-consuming induction or other non-standardized tests [42]. In a survey of over 8,500 strains conducted by 43 laboratories in the USA, the observed rates (i.e. local center results) for ceftazidime resistance in *E. cloacae* (28.4%) and *C. freundii* (31.0%) [3] were very similar to rates obtained (29.8% and 33.2%, respectively) by reference methods in the monitoring laboratory [47].

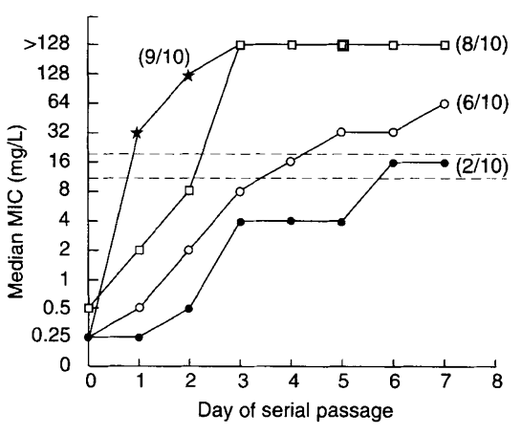

Figure 4 Median MICs for 10 *E. cloacae* strains during 7-day serial passage with a cephalosporin. The median MIC represents the sixth MIC observation when the MICs for the 10 strains on each day of testing are listed from the lowest to the highest value. The values in parentheses are the number of strains among the 10 strains tested for which the MIC was in the resistant range (≥ 32 mg/L) for the 7-day serial passage. ★ ceftriaxone; □ ceftazidime; ○ cefpirome; ● cefepime. The upper and lower broken lines in the figure are cut-offs for resistance and susceptibility (NCCLS criteria), respectively. With permission of Am Soc Microbiol J Div [46].

SIGNIFICANCE OF INDUCIBLE AND STABLY DEREPRESSED RESISTANCE

Induction potential does not necessarily translate to reduced efficacy in either the laboratory or clinical situation [48]. Confounding variables, such as the presence of multiple resistance mechanisms, outer membrane penetration, PBP affinity, enzyme inhibition by the inducer and, most importantly, the β-lactamase stability of the inducer, can affect the periplasmic concentration of the β-lactam and hence bactericidal activity. Some compounds both strongly induce and are hydrolyzed by chromosomally-mediated enzymes of Gram-negative bacteria (e.g. the aminopenicillins and the cephamycins for *E. cloacae*). Other compounds (e.g. piperacillin and other cephalosporins), although poor inducers, are labile so that greatly increased MICs are observed, despite relatively modest levels of AmpC induction. In contrast, the high AmpC-inducing potential of the carbapenems does not compromize their efficacy due to high bacterial membrane penetration and relative β-lactamase stability. The fourth-generation cephalosporins also combine high penetration rates and β-lactamase stability with low induction potential [49].

ROLE OF NEW CEPHALOSPORINS IN THERAPY

In common with third-generation cephalosporins, the fourth-generation cephalosporins have an aminothiazolyl (or amino thiadiazolyl)-methoximino group at the C-7 position of the cephem nucleus (Figure 5) [50].

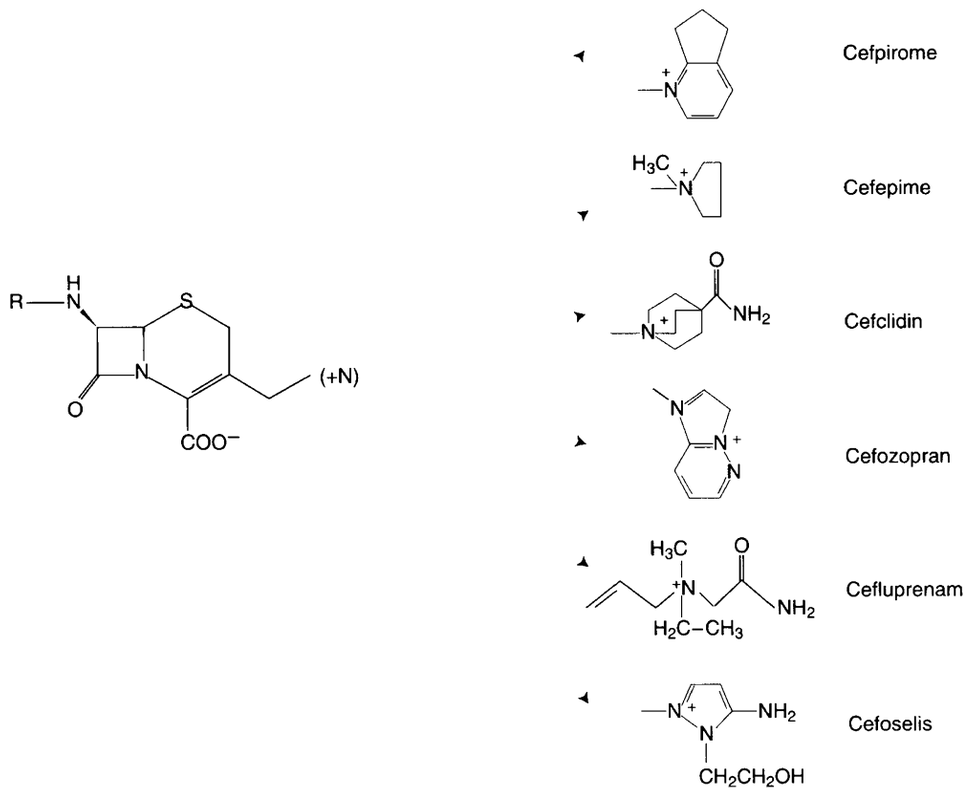

Figure 5 C-3' quaternary ammonium cephems [50].

However, these newer cephalosporins possess a quaternary ammonium group at the C-3' position which confers a considerable increase in potency and has led to these compounds being termed 'fourth-generation' cephalosporins. These C-3' substitutions confer a more balanced antimicrobial spectrum compared to ceftazidime and maintain stability to, and low affinity for, clinically important β-lactamases. They also give these compounds the properties of a zwitterion which enhances outer membrane permeability. The principal candidates for inclusion in the group are listed in Table 6 and include cefpirome and cefepime.

Both cefpirome and cefepime have been shown to penetrate the outer membrane of *E. cloacae* approximately 5- to 6-fold faster than cefotaxime. This, coupled with much lower affinity (high Km) for and higher stability towards the AmpC β-lactamase, results in higher periplasmic concentrations than those achieved by cefotaxime [51]. Consequently, MIC_{90} values of 0.5–1 mg/L are routinely achieved against *E. cloacae*, as opposed to > 32 mg/L for cefotaxime and ceftazidime [52]. Pooling of data from nine studies produced a median MIC_{90} value of 1 mg/L for cefpirome against *E. cloacae*, compared to 50 mg/L for ceftazidime [53].

Fourth-generation cephalosporins have also demonstrated excellent activity against *Enterobacter* spp. isolated from ICU [18] and other units [54]. In one study in ICU infections, cefpirome and imipenem were the most potent against ceftazidime-resistant isolates, with 94% and 97%, respectively, of strains susceptible [18]. With the exception of cefpirome, there was significant cross-resistance among the cephalosporins tested.

Fourth-generation cephalosporins generally

Table 6 List of candidate 'fourth-generation' cephalosporins for human use [50]

C-7, 2-amino-5-thiazolyl	C-7, 5-amino-2-thiadiazolyl
Cefpirome (HR-810)	Cefclidin (E-1040)
Cefepime (BMY-28142)	Cefozopran (SCE-2787)
Cefoselis (FK-037)	Cefluprenam (E-1077)

maintain good activity against ceftazidime-resistant (MIC > 16 mg/L) Enterobacteriaceae with inducible AmpC β-lactamases. In an international study of 160 ceftazidime-resistant strains [55], 74% were inhibited by cefpirome at ≤ 8 mg/L (Table 7). An identical rate of cefpirome susceptibility was noted in a five-nation survey (Table 7; Australia, France, Germany, Italy and UK) and in the USA [55]. In another 11-nation study of ceftazidime-resistant Enterobacteriaceae, > 80% of strains were inhibited by cefepime (≤ 8 mg/L), with the exception of some strains from Brazil (48%) and Italy (55%). Overall, cefpirome and cefepime display similar activities against Enterobacteriaceae which produce inducible AmpC β-lactamases, while cefocelis (FK 037) appeared slightly less active [57,58].

As yet, there are limited published clinical studies to assess the efficacy of fourth-generation cephalosporins against serious *Enterobacter* infections and especially against strains resistant to third-generation cephalosporins [59–61]. However, early indications are promising [61]. From pooled comparative clinical trials using cefepime (2,487 patient cases), 17 infections with *Enterobacter* spp. caused by organisms tested as susceptible to cefepime, but resistant to ceftazidime were observed [61]. Cefepime therapy resulted in clinical cure in all patients and an 88.2% bacteriological eradication rate. Also, no emergence of resistance was noted. In a study of 276 hospitalized patients with severe infections, three were attributable to *E. cloacae*, and were eradicated following treatment with cefpirome at 1 or 2 g bid (Table 8) [59]. In another study involving less serious infections [60], ceftazidime produced bacterial eradication in 70% of patients, whereas cefpirome at 1 g bid achieved 100% eradication (Table 8). In a Scandinavian study, cefpirome dosed at 1 g bid was found to be at least as effective as ceftazidime 1 g tid in eradicating *Citrobacter* and *Enterobacter* spp. from the urinary and respiratory tracts [62].

Another recent multicenter study compared the efficacy and safety of cefpirome and ceftazidime in the empiric treatment of nosocomial and community-acquired pneumonia in the ICU [63]. A satisfactory bacteriological response was achieved in 73% and 64% of patients receiving cefpirome (2 g bid) and ceftazidime (2 g tid), respectively, for infections caused by *Enterobacter* spp. Similarly, cefepime has demonstrated favourable results compared to ceftazidime in the treatment of infections caused by Enterobacteriaceae [64].

Table 7 Distribution of Gram-negative ceftazidime-resistant (MIC > 16 mg/L) Enterobacteriaceae strains by cefpirome MICs[a]

		Strains with following cefpirome MIC (mg/L)						
Organism	No. of strains	≤ 0.5	1	2	4	8	16	> 16
Citrobacter spp.[b]	23	6	0	6	3	5	1	2
E. cloacae	99	15	14	12	17	14	5	22
Enterobacter spp.[c]	19	12	1	1	3	1	1	0
H. alvei	7	2	0	1	1	0	0	3
M. morganii	6	2	1	0	0	0	1	2
P. stuartii	1	0	0	1	0	0	0	0
S. marcescens	5	0	0	0	0	1	0	4
Total[d]	160	37	16	21	24	21	8	33

[a]Modified from [55,56] for strains from the USA, Australia, France, Germany, Italy and the UK.
[b]Includes *Citrobacter freundii* (20 strains) and *Citrobacter* spp. (three strains, not speciated).
[c]Includes *Enterobacter aerogenes* (15 strains) and *Enterobacter* spp. (four strains, not speciated).
[d]74.4% of tested strains were susceptible (≤ 8 mg/L).

Table 8 Eradication rates for cefpirome used against infections caused by *Enterobacter* spp. [59,60]

	No. eradicated/No. treated		
Study (year)	1 g bid	2 g bid	All cases
Carbon et al. (1992)	2/2	1/1	3/3
Study group (1992)	15/15[a]	–	15/15[a]

[a]Comparator (ceftazidime) eradication rate = 70%.

ROLE OF ALTERNATIVE AGENTS

A number of alternative agents are available for the treatment of serious Gram-negative infections, although these too have resistance problems. Indeed, strains resistant to third-generation cephalosporins show a higher rate of resistance to other antibiotics of unrelated classes, such as amikacin, gentamicin and ciprofloxacin [Privitera, personal communication] (Table 9). Amongst the β-lactam antimicrobials, the carbapenems (imipenem, meropenem) have the broadest antimicrobial spectrum. Imipenem readily enters the periplasmic space of *Enterobacter* spp. via a different porin channel to that used by cephalosporins and inhibits the PBPs; it is also highly β-lactamase stable. However, clinical isolates of *Enterobacter* spp. and *P. aeruginosa* that are resistant to imipenem have been isolated recently [65]. In the USA, resistance to imipenem among Enterobacteriaceae (*Proteus* spp.) varied from 1–46%, depending on the species [66]. However, these figures also include false-positive results from some commercial systems (Vitek), emphasizing the need for in vitro monitoring methods using reference standards [3,66].

Aminoglycoside resistance continues to be a problem in the treatment of nosocomial infections. Modest increases in aminoglycoside resistance over time have occurred, even with acceptable infection control practices and therapeutic drug level monitoring. Current resistance problems with aminoglycosides include resistance mediated by reduced drug uptake in Enterobacteriaceae and *Pseudomonas* spp. and plasmid-mediated modifying enzymes (often multiple) in Enterobacteriaceae, *Pseudomonas* spp. and Gram-positive species.

Most parenteral fluoroquinolones are characterized by their broad-spectrum activity, although recent years have seen the emergence of resistant strains. Current resistance problems associated with the fluoroquinolones include resistance among methicillin-resistant *Staphylococcus aureus* (MRSA). Future problems which may become more common include resistance among *Pseudomonas* spp. and Enterobacteriaceae attributed to altered DNA topoisomerases or modified drug permeability. Ciprofloxacin resistance has been reported in *C. freundii* (9.9%), *S. marcescens* (6.8%) and *P. aeruginosa* (14.9%) in the USA in 1993–1994 [3,66] and in other countries [64].

CONCLUSIONS

Emerging resistance among Enterobacteriaceae will continue to compromize therapy with existing third-generation cephalosporins. The fourth-generation cephalosporins penetrate the bacterial outer-membrane more rapidly, have greater β-lactamase stability and, therefore, have a broader antimicrobial spectrum and higher intrinsic activity than third-generation agents. These features will sustain the class therapeutic efficacy against strains involved in serious infections in hospitalized patients.

Fourth-generation cephalosporins are active against the majority of *P. aeruginosa* and could be used as an alternative to ceftazidime as the cephalosporin of choice in combination regimens for such infections. Cefpirome and some other fourth-generation compounds have potent activity against oxacillin-susceptible staphylococci [65] and the majority of penicillin and multidrug-resistant streptococci [67,68]. Despite the improved activity and spectrum of cefpirome, it is likely that co-drugs will continue to be necessary for maximal empiric therapy of serious nosocomial infections including bacteremia, pneumonia and mixed anaerobic infections such as those in surgery patients.

Other factors, for example less frequent dosing, safety, cost and favorable interactions with other drugs (i.e. synergistic killing) will also be important factors in selecting alternative agents to complement or replace third-generation cephalosporins or other β-lactams in the treatment of infections caused by strains producing Bush Group 1 enzymes (inducible or derepressed expression).

Table 9 Association of resistance to other antimicrobial classes among 252 strains of Enterobacteriaceae having resistance to third-generation cephalosporins (Italy, 1995)

Organism	(No. tested)	% Resistance[a]		
		Amikacin	Gentamicin	Ciprofloxacin
C. freundii	(44)	13.6	31.8	28.6
E. aerogenes	(71)	24.3	15.5	48.5
E. cloacae	(100)	3.1	29.0	25.6
S. marcescens	(37)	13.9	63.9	55.2

[a]Susceptibility interpretation criteria published by the NCCLS (1995).

DISCUSSION

Prof. B. Weidemann: There may be differences in the induction potential within the cephamycin group of cephalosporins and possibly among the carbapenems, for instance, imipenem has a greater induction potential than meropenem.

Prof. F. Baquero: It remains unclear whether differences in the induction potential between strains of a particular species are important. For cefpirome, the low induction can be partially explained by the rapid bactericidal activity, as both cefoxitin and cefpirome are equally effective against the cell wall. The inducer is produced at the same rate for both cephalosporins, therefore, the observed differences are related to the relative speeds of killing; rather than differences in induction potential.

Prof. B. Wiedemann: Differences in the induction potential of the drugs are related to binding to PBP 5. The stronger the binding to PBP 5, then the more intense the induction.

Prof. K. Klugman: The increasing worldwide importance of the extended-spectrum β-lactamases (ESBLs), should not be overlooked, particularly regarding the impact on MIC values.

Prof. R. Jones: Yes I agree, the overall pattern of emerging resistance in *E. coli*, or *Klebsiella* spp. is going to mimic the pattern among stably-derepressed β-lactamase-producing *Enterobacter* or *Citrobacter* to the clinical microbiologist. Would Dr Bauernfeind address this issue?

Dr. A. Bauernfeind: To be more specific, the incidence of AmpC genes on plasmids is increasing world-wide. However, one advantage of the fourth-generation cephalosporins is that they retain good in vitro activity against *ampC* plasmid containing strains.

Prof. F. Baquero: The activity against plasmid mediated *ampC* producing Enterobacteriaceae is a potential advantage for the fourth-generation cephalosporins.

Prof. R. Jones: The number of strains with ESBL phenotypes is becoming alarmingly high in the USA. Dr Pfaller, do you have any comment on this?

Dr. M. Pfaller: Recent data demonstrate that ≥ 40% of *Klebsiella* spp. in individual institutions are ESBL-producing strains. Not all these strains are the result of an outbreak of a single clone, and the percentage varies from one institution to another and between strains in the same medical center. There is considerable variation in the incidence of ESBLs and the incidence should be closely monitored.

Prof. R. Jones: In hospitals with a high incidence of ESBL phenotypes, approximately 50% of strains are cefoxitin-resistant, often carrying multiple resistance phenotypes. This appears to be due to mobilization of the *ampC* gene into *K. pneumoniae*. Approximately 17% of current bacteremias in a large hospital sample (60 medical centers) in the USA, due to *K. pneumoniae*, are ESBL or *ampC* phenotypes.

Prof. F. Baquero: In the study by Dr. E. Sanders, the emergence of *ampC* mutants were not detected following a 1 g bid dose of cefepime. In an analysis of the ceftazidime-resistant strains a trimodal MIC distribution was observed for cefepime; one peak was at 0.5 mg/L, one at about 4 mg/L and one at ≥ 8 mg/L. These strains may also exhibit increased MIC values the carbapenems.

Prof. R. Jones: Examination of the susceptibility testing data demonstrates that the usual cefepime MIC was in the 'first mode' (previously mentioned). All the fourth-generation cephalosporins tested against these ceftazidime-resistant strains exhibit a trimodal effect, although there is variation of the MIC values of particular agents.

Prof. J. Turnidge: The main problem with the emergence of resistance is with *Enterobacter cloacae*, which is the most prevalent of pathogens and also seems to have the highest propensity for the development of resistance.

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